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(54) Title: PURIFIED SCYTALIDIUM LACCASES AND NUCLEIC ACIDS ENCODING SAME (57) Abstract The present invention relates to isolated nucleic acid constructs containing a sequence encoding a <i>Scytalidium</i> laccase, and the laccase proteins encoded thereby.		

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PURIFIED SCYTALIDIUM LACCASES AND NUCLEIC ACIDS ENCODING SAME

5

Field of the Invention

The present invention relates to isolated nucleic acid fragments encoding a fungal oxidoreductase enzyme and the
10 purified enzymes produced thereby. More particularly, the invention relates to nucleic acid fragments encoding a phenol oxidase, specifically a laccase, of a thermophilic fungus, *Scytalidium*.

15 Background of the Invention

Laccases (benzenediol:oxygen oxidoreductases) are multi-copper containing enzymes that catalyze the oxidation of phenolics. Laccase-mediated oxidations result in the production of aryloxy-radical intermediates from suitable
20 phenolic substrate; the ultimate coupling of the intermediates so produced provides a combination of dimeric, oligomeric, and polymeric reaction products. Such reactions are important in nature in biosynthetic pathways which lead to the formation of melanin, alkaloids, toxins, lignins, and
25 humic acids. Laccases are produced by a wide variety of fungi, including ascomycetes such as *Aspergillus*, *Neurospora*, and *Podospora*, the deuteromycete *Botrytis*, and basidiomycetes such as *Collybia*, *Fomes*, *Lentinus*, *Pleurotus*, *Trametes*, and perfect forms of *Rhizoctonia*. Laccase
30 exhibits a wide range of substrate specificity, and each different fungal laccase usually differs only quantitatively from others in its ability to oxidize phenolic substrates. Because of the substrate diversity, laccases generally have found many potential industrial applications. Among these

are lignin modification, paper strengthening, dye transfer inhibition in detergents, phenol polymerization, juice manufacture, phenol resin production, and waste water treatment.

- 5 Although the catalytic capabilities are similar, laccases made by different fungal species do have different temperature and pH optima, and these may also differ depending on the specific substrate. A number of these fungal laccases have been isolated, and the genes for
10 several of these have been cloned. For example, Choi et al. (Mol. Plant-Microbe Interactions 5: 119-128, 1992) describe the molecular characterization and cloning of the gene encoding the laccase of the chestnut blight fungus, *Cryphonectria parasitica*. Kojima et al. (J. Biol. Chem.
15 265: 15224-15230, 1990; JP 2-238885) provide a description of two allelic forms of the laccase of the white-rot basidiomycete *Coriolus hirsutus*. Germann and Lerch (Experientia 41: 801, 1985; PNAS USA 83: 8854-8858, 1986) have reported the cloning and partial sequencing of the
20 *Neurospora crassa* laccase gene. Saloheimo et al. (J. Gen. Microbiol. 137: 1537-1544, 1985; WO 92/01046) have disclosed a structural analysis of the laccase gene from the fungus *Phlebia radiata*.

- Attempts to express laccase genes in heterologous
25 fungal systems frequently give very low yields (Kojima et al., *supra*; Saloheimo et al., Bio/Technol. 9: 987-990, 1991). For example, heterologous expression of *Phlebia radiata* laccase in *Trichoderma reesei* gave only 20 mg per liter of active enzyme (Saloheimo, 1991, *supra*). Although
30 laccases have great commercial potential, the ability to express the enzyme in significant quantities is critical to their commercial utility. At the present time there are no laccases which are expressed at high levels in commercially utilized hosts such as *Aspergillus*. Thus, the need exists

for a laccase which can be produced in commercially useful (i.e., gram per liter or more) quantities. The present invention fulfills such a need.

5 Summary of the Invention

The present invention relates to a DNA construct containing a nucleic acid sequence encoding a *Scytalidium* laccase. The invention also relates to an isolated laccase encoded by the nucleic acid sequence. Preferably, the
10 laccase is substantially pure. By "substantially pure" is meant a laccase which is essentially (i.e., ≥90%) free of other non-laccase proteins.

In order to facilitate production of the novel laccase, the invention also provides vectors and host cells
15 comprising the claimed nucleic acid fragment, which vectors and host cells are useful in recombinant production of the laccase. The nucleic acid fragment is operably linked to transcription and translation signals capable of directing expression of the laccase protein in the host cell of
20 choice. A preferred host cell is a fungal cell, most preferably of the genus *Aspergillus*. Recombinant production of the laccase of the invention is achieved by culturing a host cell transformed or transfected with the nucleic acid fragment of the invention, or progeny thereof, under
25 conditions suitable for expression of the laccase protein, and recovering the laccase protein from the culture.

The laccases of the present invention are useful in a number of industrial processes in which oxidation of phenolics is required. These processes include lignin
30 manipulation, juice manufacture, phenol polymerization and phenol resin production.

Brief Description of the Figures

Figure 1 illustrates the nucleotide (SEQ ID NO: 1) and amino acid (SEQ ID NO: 2) sequence of *Scytalidium thermophila* laccase. Letters without corresponding amino acids in the nucleotide sequence indicate the position of
5 introns.

Figure 2 illustrates the construction of plasmid pShTh15.

Figure 3 illustrates the restriction map of a *XhoI* insert in pShTh6 which contains the *S. thermophilum*
10 laccase (*lccS*) gene. The approximate position of the *lccS* coding region is indicated by a solid black line.

Figure 4 illustrates the pH profiles of the laccase activity with syringaldazine (squares) and 2,2' azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (circles) as substrate.
15 Figure 5 illustrates the thermostability in B&R buffers of the laccase at pH 2.7, 6.1, and 9.0. Preincubation times are 1 hour. Activities are assayed by ABTS oxidation at 20°C in B&R buffer, pH 4.1.

20 Detailed Description of the Invention

Scytalidium thermophilum is a thermophilic deuteromycete, and a member of the *Torula-Humicola* complex which are recognized as dominant species in mushroom compost. Other members of the complex include *Humicola*
25 *grisea* Traaen var. *thermoidea* Cooney & Emerson, *H. insolens* Cooney & Emerson, and *Torula thermophila* Cooney & Emerson, the latter of which has been reassigned to *Scytalidium thermophilum* by Austwick (N.Z. J. Agric. Res. 19: 25-33, 1976). Straatsma and Samson (Mycol. Res. 97: 321-328, 1993)
30 have recently determined that both *H. grisea* var. *thermoidea* and *H. insolens* should be considered as examples of the species *Scytalidium thermophilum* as well. *S. indonesiacum* (Hedger et al., Trans. Brit Mycol. Soc. 78: 366-366, 1982) may also be synonymous with *S. thermophilum*. Members of the

complex are known to be producers of thermostable cellulase and β -glucosidase enzymes (Rao and Murthy, Ind. J. Biochem. Biophys. 25: 687-694, 1988; Hayashida and Yoshioka, Agric. Biol. Chem. 44: 1721-1728, 1980). However, there have been
5 no previous reports of the production of a laccase by *Scytalidium*, or any of the noted synonymous species. It has now been determined that not only does *Scytalidium* produce a laccase, but the gene encoding this laccase can be used to produce large yields of the enzyme in convenient host
10 systems such as *Aspergillus*.

To identify the presence of a laccase gene in *Scytalidium*, a 5' portion of the *Neurospora crassa* laccase gene (*lcc1*) is used as a probe, under conditions of mild stringency, in southern hybridization of total genomic DNA
15 of different fungal species. An approximately 3 kb laccase specific sequence is detected in the *Scytalidium* DNA. The *N. crassa* fragment is then used to screen about 12,000 plaques of an *S. thermophilum* genomic DNA library in a λ EMBL4 bacteriophage cloning vector. Nine plaques strongly
20 hybridize with the probe; from these nine, DNA is isolated from four. Each of these clones contains a 3kb *Bam*HI fragment corresponding to the one initially identified in the southern blot of genomic DNA. One of the fragments is subcloned into a pBluescript vector; however, DNA sequencing
25 shows only a portion of the gene to be on this fragment. A 6kb fragment *Xho*I fragment from the same phage contains the whole *lccS* gene, and this is then subcloned into pBluescript to derive plasmid pShTh6. A restriction map of the 6 kb insert is shown in Figure 3.

30 Once the sequence is determined, the positions of introns and exons within the gene is assigned based on alignment of the deduced amino acid sequence to the corresponding *N. crassa* laccase gene product. From this comparison, it appears that the gene (*lccS*) of *S.*

thermophilum is composed of seven exons (243, 91, 70, 1054 and 390 nucleotides) punctuated by four small introns (63, 58, 55 and 65 nucleotides). The coding region, excluding intervening sequences is very GC-rich (60.8% G+C) and encodes
5 a preproenzyme of 616 amino acids: a 21 amino acid signal peptide and a 24 amino acid propeptide. The sequence of the *S. thermophilum* gene and the predicted amino acid sequence is shown in Figure 1 (SEQ ID NOS: 1 and 2)

The laccase gene is then used to create an expression
10 vector for transformation of *Aspergillus* host cells. The vector, pShTh15 contains the *A. oryzae* TAKA-amylase promoter and the *A. niger glaA* terminator regions. The construction of pShTh15 is outlined in Figure 2. *Aspergillus* cells are cotransformed with the expression vector and a plasmid
15 containing the *pyrG* or *amdS* selectable marker. Transformants are selected on the appropriate selective medium containing ABTS. Laccase-producing colonies exhibit a green halo and are readily isolatable. Selected transformants are grown up in shake flasks and culture
20 broths tested for laccase activity by the syringaldazine method. Shake flask cultures are capable of producing 50 or more mg/liter of laccase, and in fermentors, yields of over 1.6 g/liter are observed.

According to the invention, a *Scytalidium* gene
25 encoding a laccase can be obtained by methods described above, or any alternative methods known in the art, using the information provided herein. The gene can be expressed, in active form, using an expression vector. A useful expression vector contains an element that permits stable
30 integration of the vector into the host cell genome or autonomous replication of the vector in a host cell independent of the genome of the host cell, and preferably one or more phenotypic markers which permit easy selection of transformed host cells. The expression vector may also

include control sequences encoding a promoter, ribosome binding site, translation initiation signal, and, optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides
5 encoding a signal sequence may be inserted prior to the coding sequence of the gene. For expression under the direction of control sequences, a laccase gene to be used according to the invention is operably linked to the control sequences in the proper reading frame. Promoter
10 sequences that can be incorporated into plasmid vectors, and which can direct the transcription of the laccase gene, include but are not limited to the prokaryotic β -lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et
15 al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; and in Sambrook et al., Molecular Cloning, 1989.

The expression vector carrying the DNA construct of the
20 invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will typically depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an
25 extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host
30 cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in

the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA construct of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis* α -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus amyloliquefaciens* α -amylase (*amyQ*), or the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes. In a yeast host, a useful promoter is the *eno-1* promoter. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α -amylase, *A. niger* acid stable α -amylase, *A. niger* or *A. awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase. Preferred are the TAKA-amylase and *glaA* promoters.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the laccase of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter. The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of replication of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from *B.subtilis* or *B.licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Examples of *Aspergillus* selection markers include *amdS*, *pyrG*, *argB*, *niaD*, *sc*, and *hygB* a marker giving rise to hygromycin resistance. Preferred for use in an *Aspergillus* host cell are the *amdS* and *pyrG* markers of *A. nidulans* or *A. oryzae*. A frequently used mammalian marker is the dihydrofolate reductase (DHFR) gene. Furthermore, selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

It is generally preferred that the expression gives rise to a product that is extracellular. The laccases of the present invention may thus comprise a preregion permitting secretion of the expressed protein into the culture medium. If desirable, this preregion may be native to the laccase of the invention or substituted with a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions. For example, the preregion may be derived from a glucoamylase or an amylase gene from an *Aspergillus* species, an amylase gene from a *Bacillus* species, a lipase or proteinase gene from *Rhizomucor miehei*, the gene for the α -factor from *Saccharomyces cerevisiae* or the calf preprochymosin gene. Particularly preferred, when the host is a fungal cell, is the preregion for *A. oryzae* TKA amylase, *A. niger* neutral amylase, the maltogenic amylase form *Bacillus* NCIB 11837, *B. stearothermophilus* α -amylase, or *Bacillus licheniformis* subtilisin. An effective signal sequence is the *A. oryzae* TKA amylase signal, the

Rhizomucor miehei aspartic proteinase signal and the *Rhizomucor miehei* lipase signal.

The procedures used to ligate the DNA construct of the invention, the promoter, terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. Molecular Cloning, 1989).

10 The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in the recombinant production of a enzyme of the invention. The cell may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

25 The host cell may be selected from prokaryotic cells, such as bacterial cells. Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may for instance be effected

by protoplast transformation or by using competent cells in a manner known *per se*.

The host cell may also be a eukaryote, such as mammalian cells, insect cells, plant cells or preferably
5 fungal cells, including yeast and filamentous fungi. For example, useful mammalian cells include CHO or COS cells. A yeast host cell may be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. Useful filamentous fungi may selected from a
10 species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Alternatively, a strain of a *Fusarium* species, e.g. *F. oxysporum*, can be used as a host cell. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus*
15 host cells is described in EP 238 023. A suitable method of transforming *Fusarium* species is described by Malardier et al., 1989.

20 The present invention thus provides a method of producing a recombinant laccase of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the enzyme and recovering the enzyme from the cells and/or culture
25 medium. The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the laccase of the invention. Suitable media are available from commercial suppliers or may be prepared according to published formulae
30 (e.g. in catalogues of the American Type Culture Collection).

The resulting enzyme may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, precipitat-

ing the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, gel filtration

- 5 chromatography, affinity chromatography, or the like. Preferably, the isolated protein is about 90% pure as determined by SDS-PAGE, purity being most important in food, juice or detergent applications.

In a particularly preferred embodiment, the expression
10 of laccase is achieved in a fungal host cell, such as *Aspergillus*. As described in detail in the following examples, the laccase gene is ligated into a plasmid containing the *Aspergillus oryzae* TAKA α -amylase promoter, and the *Aspergillus nidulans* *amdS* selectable marker.
15 Alternatively, the *amdS* may be on a separate plasmid and used in co-transformation. The plasmid (or plasmids) is used to transform an *Aspergillus* species host cell, such as *A. oryzae* or *A. niger* in accordance with methods described in Velton et al. (PNAS USA 81: 1470-1474, 1984).

- 20 Those skilled in the art will recognize that the invention is not limited to use of the nucleic acid fragments specifically disclosed herein, for example, in Figure 1. It will also be apparent that the invention encompasses those nucleotide sequences that encode the same
25 amino acid sequences as depicted in Figure 1, but which differ from those specifically depicted nucleotide sequences by virtue of the degeneracy of the genetic code. Also, reference to Figure 1, in the specification and the claims will be understood to encompass both the genomic sequence
30 depicted therein as well as the corresponding cDNA and RNA sequences, and the phrases "DNA construct" and "nucleic acid sequences" as used herein will be understood to encompass all such variations. "DNA construct" shall generally be understood to mean a DNA molecule, either single- or double-

stranded, which may be isolated in partial form from a naturally occurring gene or which has been modified to contain segments of DNA which are combined and juxtaposed in a manner which would not otherwise exist in nature.

5 In addition, the invention also encompasses other *Scytalidium* laccases, including alternate forms of laccase which may be found in *S. thermophilum* and as well as laccases which may be found in other fungi which are synonyms or fall within the definition of *Scytalidium*
10 *thermophilum* as defined by Straatsma and Samson, 1993, *supra*. These include *S. indonesiacum*, *Torula thermophila*, *Humicola brevis* var. *thermoidea*, *Humicola brevispora*, *H. grisea* var. *thermoidea*, *Humicola insolens*, and *Humicola lanuginosa* (also known as *Thermomyces lanuginosus*). The
15 invention also provides the means for isolation of laccase genes from other species of *Scytalidium*, such as *S. acidophilum*, *S. album*, *S. aurantiacum*, *S. circinatum*, *S. flaveobrunneum*, *S. hyalinum*, *S. lignicola*, and *S. uredinicolum*. Identification and isolation of laccase genes
20 from sources other than those specifically exemplified herein can be achieved by utilization of the methodology described in the present examples, with publicly available *Scytalidium* strains. Alternately, the sequence disclosed herein can be used to design primers and/or probes useful in
25 isolating laccase genes by standard PCR or southern hybridization techniques, using the same publicly available strains. Examples of such publicly available strains include, from the American Type Culture Collection, ATCC 16463, 28085, 36346, 48409, 66938 (*S. thermophilum*); 24569 (*S.*
30 *acidophilum*); 16675 (*S. album*); 22477 (*S. aurantiacum*); 66463 (*S. circinatum*); 13212 (*S. flavo-brunneum*); 52297 (*S. fulvum*); 38906 (*S. hyalinum*); 46858 (*S. indonesiacum*); 18984 (*S. indonesiacum*); 32382 (*S. uredinaolum*); from the International Mycological Institute (IMI; United Kingdom),

IMI 243 118(*S. thermophilum*); from Centraalbureau voor Schimmelcultures(CBS; Netherlands) CBS 183.81, 671.88(*S. thermophilum*) 367.72(*S. acidophilum*); 372.65(*S. album*);374.65(*S. aurantiacum*);654.89(*S. circinatum*);244.59
5 (*S. flavo-brunneum*);145.78 (*S. hyalinum*);259.81(*S. indonesiacum*);233.57(*S. lignicola*);171.40(*S. terminale*);
616.84(*S. muscorum*); from Deutsche Sammlung von Mikroorganismenn und Zellkulturen (DSM; Germany) DSM 2842(*S. thermophilum*); DSM 2695 (*S. lignicola*). The invention also
10 encompasses any variant nucleotide sequence, and the protein encoded thereby, which protein retains at least about an 80%, preferably about 85%, and most preferably at least about 90-95% homology with the amino acid sequence depicted in Figure 1, and which qualitatively retains the laccase
15 activity of the sequence described herein. Useful variants within the categories defined above include, for example, ones in which conservative amino acid substitutions have been made, which substitutions do not significantly affect the activity of the protein. By conservative substitution is
20 meant that amino acids of the same class may be substituted by any other of that class. For example, the nonpolar aliphatic residues Ala, Val, Leu, and Ile may be interchanged, as may be the basic residues Lys and Arg, or the acidic residues Asp and Glu. Similarly, Ser and Thr are
25 conservative substitutions for each other, as are Asn and Gln. It will be apparent to the skilled artisan that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active enzyme. Retention of the desired activity can readily be
30 determined by conducting a standard ABTS oxidation method, such as is described in the present examples.

The protein can be used in number of different industrial processes. These processes include polymerization of lignin, both Kraft and liginosulfates, in

solution, in order to produce a lignin with a higher molecular weight. A neutral/alkaline laccase is a particular advantage in that Kraft lignin is more soluble at higher pHs. Such methods are described in, for example, Jin et al., *Holzforschung* 45(6): 467-468, 1991; US Patent No. 4,432,921; EP 0 275 544; PCT/DK93/00217, 1992. Laccase is also useful in the copolymerization of lignin with low molecular weight compounds, such as is described in Appl. Microbiol. Biotechnol. 40: 760-767.

10 The laccase of the present invention can also be used for in-situ depolymerization of lignin in Kraft pulp, thereby producing a pulp with lower lignin content. This use of laccase is an improvement over the current use of chlorine for depolymerization of lignin, which leads to the
15 production of chlorinated aromatic compounds, which are an environmentally undesirable by-product of paper mills. Such uses are described in, for example, Current opinion in Biotechnology 3: 261-266, 1992; J. Biotechnol. 25: 333-339, 1992; Hiroi et al., *Svensk papperstidning* 5: 162-166, 1976.
20 Since the environment in a paper mill is typically alkaline, the present laccase is more useful for this purpose than other known laccases, which function best under acidic conditions.

 Oxidation of dyes or dye precursors and other
25 chromophoric compounds leads to decolorization of the compounds. Laccase can be used for this purpose, which can be particularly advantageous in a situation in which a dye transfer between fabrics is undesirable, e.g., in the textile industry and in the detergent industry. Methods for
30 dye transfer inhibition and dye oxidation can be found in WO 92/01406; WO 92/18683; EP 0495836; Calvo, Mededelingen van de Faculteit Landbouw-wetenschappen/Rijksuniversitet Gent. 56: 1565-1567, 1991; Tsujino et al., J. Soc. Chem. 42: 273-282, 1991. Use of laccase in oxidation of dye precursors

for hair dyeing is disclosed in U.S. Patent No. 3,251,742, the contents of which are incorporated herein by reference.

The present laccase can also be used for the polymerization or oxidation of phenolic compounds present in liquids. An example of such utility is the treatment of juices, such as apple juice, so that the laccase will accelerate a precipitation of the phenolic compounds present in the juice, thereby producing a more stable juice. Such applications have been described in Stutz, Fruit processing 7/93, 248-252, 1993; Maier et al., Dt. Lebensmittel-rindschau 86(5): 137-142, 1990; Dietrich et al., Fluss. Obst 57(2): 67-73, 1990.

Laccases such as the *Scytalidium* laccase are also useful in soil detoxification (Nannipieri et al., J. Environ. Qual. 20: 510-517, 1991; Dec and Bollag, Arch. Environ. Contam. Toxicol. 19: 543-550, 1990).

The invention is further illustrated by the following non-limiting examples.

EXAMPLES

20

I. ISOLATION OF SCYTALIDIUM THERMOPHILUM LACCASE GENE

A. MATERIALS AND METHODS

1. DNA Extraction and Hybridization analysis

25 Total cellular DNA is extracted from fungal cells of *Scytalidium thermophila* strain E421 grown 24 hours in 25 ml of YEG medium (0.5% yeast extract, 2% glucose) using the following protocol: Mycelia are collected by filtration through Miracloth (Calbiochem) and washed once with 25 ml of TE buffer. Excess buffer is drained from the mycelia which are subsequently frozen in liquid nitrogen. Frozen mycelia are ground to a fine powder in an electric coffee grinder, and the powder added to 20 ml of TE buffer and 5 ml of 20% SDS (w/v) in a disposable plastic centrifuge tube.

The mixture is gently inverted several times to ensure mixing, and extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Sodium acetate (3M solution) is added to give a final concentration of 0.3 M and the nucleic acids are precipitated with 2.5 volumes of ice cold ethanol. The tubes are centrifuged at 15,000 x g for 30 minutes and the pellet is allowed to air-dry for 30 minutes before resuspending in 0.5 ml of TE buffer. DNase-free ribonuclease A is added to a concentration of 100µg/ml and the mixture is incubated at 37°C for 30 minutes. Proteinase K (200µg/ml) is added and each tube is incubated an additional one hour at 37°C. Finally, each sample is extracted twice with phenol:chloroform:isoamyl alcohol before precipitating the DNA with sodium acetate and ethanol. DNA pellets are dried under vacuum, resuspended in TE buffer, and stored at 4°C.

Total cellular DNA samples are analyzed by Southern hybridization. Approximately 5µg of DNA is digested with *EcoRI* and fractionated by size on a 1% agarose gel. The gel is photographed under short wavelength UV and soaked for 15 minutes in 0.5 M NaOH, 1.5 M NaCl followed by 15 minutes in 1 M Tris-HCl, pH 8, 1.5 M NaCl. DNA in the gel is transferred onto Zeta-Probe™ hybridization membrane (BioRad Laboratories) by capillary blotting in 20 X SSPE (R. W. Davis et al., Advanced Bacterial Genetics, A Manual for Genetic Engineering. Cold Spring Harbor Press. 1980) Membranes are baked for 2 hours at 80°C under vacuum and soaked for 2 hours in the following hybridization buffer at 45°C with gentle agitation: 5X SSPE, 35% formamide (v/v), 0.3% SCS, 200µg/ml denatured and sheared salmon testes DNA. The laccase-specific probe fragment (approx. 1.5 kb) encoding the 5'-portion of the *N. crassa lcc1* gene is amplified from *N. crassa* genomic DNA using standard PCR conditions (Perkin-Elmer Cetus, Emeryville, CA) with the

following pair of primers: forward primer, 5'
CGAGACTGATAACTGGCTTGG 3'; reverse primer, 5'
ACGGCGCATTGTCAGGGAAGT 3'. The amplified DNA segment is
first cloned into a TA-cloning vector (Invitrogen, Inc., San
5 Diego, CA), then purified by agarose gel electrophoresis
following digestion with *EcoRI*. The purified probe fragment
is radiolabeled by nick translation with α [³²P]dCTP (Amersham)
and added to the hybridization buffer at an activity of
approximately 1 X 10⁶ cpm per ml of buffer. The mixture is
10 incubated overnight at 45°C in a shaking water bath.
Following incubation, the membranes are washed once in 0.2 X
SSPE with 0.1% SDS at 45°C followed by two washes in 0.2 X
SSPE (no SDS) at the same temperature. The membranes are
allowed to dry on paper towels for 15 minutes, then wrapped
15 in Saran Wrap™ and exposed to x-ray film overnight at -70°C
with intensifying screens (Kodak).

2. DNA Libraries and Identification of Laccase Clones

Genomic DNA libraries are constructed in the
bacteriophage cloning vector λ -EMBL4 (J.A. Sorge, in Vectors,
20 A Survey of Molecular Cloning Vectors and Their Uses,
Rodriguez et al., eds, pp.43-60, Butterworths, Boston,
1988). Briefly, total cellular DNA is partially digested
with *Sau3A* and size-fractionated on low-melting point
agarose gels. DNA fragments migrating between 9kb and 23 kb
25 are excised and eluted from the gel using β -agarase (New
England Biolabs, Beverly MA). The eluted DNA fragments are
ligated with *Bam*HI-cleaved and dephosphorylated λ -EMBL4
vector arms, and the ligation mixtures are packaged using
commercial packaging extracts (Stratagene, LaJolla, CA).
30 The packaged DNA libraries are plated and amplified on
Escherichia coli K802 cells. Approximately 10,000-20,000
plaques from each library are screened by plaque-
hybridization with the radiolabeled *lcc1* DNA fragment using

the conditions described above. Plaques which give hybridization signals with the probe are purified twice on *E. coli* K802 cells, and DNA from the corresponding phage is purified from high titer lysates using a Qiagen Lambda kit (Qiagen, Inc., Chatsworth, CA).

3. Analysis of Laccase Genes

Restriction mapping of laccase clones is done using standard methods (Lewin, Genes. 2d ed., Wiley & Sons, 1985, New York). DNA sequencing is done with an Applied Biosystems Model 373A automated DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) using the primer walking technique with dye-terminator chemistry (H. Giesecke et al., J. Virol. Methods 38: 47-60, 1992). Oligonucleotide sequencing primers are synthesized on an Applied Biosystems model 394 DNA/RNA Synthesizer.

B. RESULTS AND DISCUSSION

1. Identification of Laccase Gene Sequence

Total cellular DNA samples are prepared from the species *Neurospora crassa*, *Botrytis cinerea*, and *Scytalidium*. Aliquots of these DNA preparations are digested with *Bam*HI and fractionated by agarose gel electrophoresis. DNA in the gel is blotted to a Zeta-Probe™ membrane filter (BioRad Laboratories, Hercules, CA) and probed under conditions of mild stringency with a radiolabeled fragment encoding a portion of the *N. crassa lcc1* gene, as described above. Laccase-specific sequences are detected in the genomes of *S. thermophilum* and the *N. crassa* control, but not in the *B. cinerea* genomic DNA with this probe.

2. Cloning and Characterization of *Scytalidium thermophila* Laccase (StL) Gene

The *S. thermophilum* laccase gene is isolated using plaque hybridization to screen the genomic DNA library made

in λ -EMBL4. The library contains approximately 250,000 independent clones before amplification, and 12,000 plaques are screened by hybridization with a radiolabeled *N. crassa* laccase gene fragment as described above. Nine plaques are identified which hybridize strongly to the probe. DNA is isolated from four of these clones and analyzed by restriction mapping. All four contain a 3kb *Bam*HI fragment that is originally identified in southern blotting with genomic DNA as described above. This fragment is isolated from one clone and inserted into a pBluescript vector (Stratagene Cloning Systems, La Jolla, CA). However, DNA sequence analysis indicates that only a portion of the gene is located on this segment. Consequently, a 6 kb *Xho*I fragment which contains the entire *lccS* gene is subcloned into pBluescript to derive the plasmid pShTh6. A restriction map of the 6 kb insert in this plasmid is shown in Figure 3. The nucleic acid sequence is shown in Figure 1 and SEQ ID NO: 1. The deduced amino acid sequence of StL is obtained on the basis of amino acid sequence homology with *N. crassa* laccase. StL shares approximately 58% amino acid sequence identity with NcL, and this sequence similarity is highest among those amino residues that are involved in the formation of the active site copper center. StL, like NcL appears to be synthesized as a preproenzyme (616 amino acids with a 21 amino acid signal peptide and a propeptide of 24 amino acids). However, since the amino terminal sequence of the mature StL protein is not yet determined, the exact length of the propeptide is not certain. There are five potential sites for N-linked glycosylation in StL. A potential C-terminal processing signal with homology to *N. crassa* laccase also exists in StL (Asp-Ser-Gly-Leu*⁵⁶⁴Lys) which may result in the proteolytic removal of the last seven amino acids from the primary translation product.

The presence of four small introns (63, 58, 55 and 65 nucleotides) is determined by comparing the open reading frames within the coding region of *lccS* to the primary structure of NcL. Excluding these intervening sequences, the coding region contains 60.8% G+C. The base composition of *lccS* reflects a bias for codons ending in G or C.

II. EXPRESSION OF SCYTALIDIUM LACCASE IN ASPERGILLUS

10 A. MATERIALS AND METHODS

1. Bacterial and Fungal Host Strains

Escherichia coli JM101 (Messing et al., Nucl. Acids Res. 9:309-321, 1981) is used as a host for construction and routine propagation of laccase expression vectors in this study. Fungal hosts for laccase expression included the *Aspergillus niger* strain Bo-1, as well as a uridine-requiring (*pyrG*) mutant of the α -amylase-deficient *Aspergillus oryzae* strain HowB104.

2. Plasmids

20 Plasmid pShTh5 is a pBluescript (Stratagene Cloning Systems, LaJolla, CA) derivative which contains a 6kb *Xho*I fragment of *S. thermophilum* DNA encoding StL. Plasmid pToC68 (WO 91/17243) contains the *A. oryzae* TAKA-amylase promoter and *A. niger glaA* terminator, and pToC90 (WO 25 91/17243) carries the *A. nidulans amdS* gene.

3. Construction of Laccase Expression Vectors

The construction strategy for the laccase expression vector pShTh15 is outlined in Figure 2. The promoter directing transcription of the laccase gene is obtained from 30 the *A. oryzae* α -amylase (TAKA-amylase) gene (Christensen et al., *supra*), and terminator from the *A. niger glaA* (glucoamylase) terminator region. The expression vector is constructed as follows. A 60 basepair synthetic DNA linker,

5' TCGAGATGAAGCGCTTCTTCATTAATAGCCTTCTGCTTCTCGCAGGGCTCCTCAACTCAGGGGCC 3'
 3' CTACTTCGCGAAGAAGTAATTATCGGAAGACGAAGAGCGTCCCGAGGAGTTGAGTCC 5'

5 including the region from start codon to an *ApaI* site, is inserted into *XhoI*- and *ApaI*-digested pBluescriptSK- (Stratagene, LaJolla, CA) to produce an intermediate termed pShTh11.5. This vector is digested with *ApaI* and *Asp718* and ligated with a 662 base pair *ApaI*-*Asp718* fragment encoding a
 10 portion of StL from pShTh5, generating a second intermediate called pShTh13.1. An *XbaI* site is introduced immediately downstream of the stop codon using pShTh5 as a template for a PCR reaction with the following primers: forward:
 5'GTCATGAACAATGACCT 3'; reverse:
 15 5'AGAGAGTCTAGATTAAACAATCCGCCCAACTAC3'. The amplified fragment is digested with *NsiI* and *XbaI* and subcloned into pUC518 to created the intermediate called pShTh12.8. The pShTh12.8 vector is digested with *EcoRI* and *Asp718* and ligated with a 700 base pair *EcoRI*-*Asp718* fragment from pShTh13.1 to generate
 20 pShTh13.1 to generate pShTh13.2. An 800 base pair *NsiI*-*Asp718* fragment containing the final portion of the laccase coding region is obtained from pShTh5 and inserted into *NsiI*- and *Asp718*-cleaved pShTh13.2 to give pShTh14. Lastly, the 2.2 kb laccase coding region in pShTh14 is removed by cleavage with
 25 *XhoI* and *XbaI* and inserted between the *XhoI* and *XbaI* sites of pToC68 to generate the expression vector pShTh15.

4. Transformation of *Aspergillus* host cells

Methods for co-transformation of *Aspergillus* strains are as described in Christensen et al., *supra*. For introduction
 30 of the laccase expression vectors into *A. oryzae* HowB 104 *pyrG*, equal amounts (approximately 5 µg each) of laccase expression vector and pPyrG, which harbors the cloned *A. nidulans pyrG* gene, are used. Protrophic(Pyr⁺) transformants are selected on *Aspergillus* minimal medium

(Rowlands and Turner, Mol. Gen. Genet. 126: 201-216, 1973), and the transformants are screened for the ability to produce laccase on minimal medium containing 1 mM 2,2'-azinobis(3-ethylbenzthiazolinesulfonic acid) [ABTS]. Cells which secrete
5 active laccase oxidize the ABTS, producing a green halo surrounding the colony. *A. niger* Bo-1 protoplasts are co-transformed using equal amounts (approximately 5µg each) of laccase expression vector and pToC90 which contains the *A. nidulans amdS* (acetamidase) gene (Hynes et al., Mol. Cell
10 Biol. 3: 1430-1439, 1983. *AmdS*⁺ transformants are selected on Cove minimal medium (Cove, Biochim. Biophys. Acta 113: 51-56, 1966) with 1% glucose as the carbon source and acetamide as the sole nitrogen source and screened for laccase expression on Cove medium with 1 mM ABTS.

15 5. Analysis of Laccase-Producing Transformants

Transformants which produce laccase activity on agar plates are purified twice through conidiospores and spore suspensions in sterile 0.01% Tween-80 are made from each. The density of spores in each suspension is estimated
20 spectrophotometrically (A_{595} nm). Approximately 0.5 absorbance units of spores are used to inoculate 25 ml of ASPO4 or MY50 medium in 125 ml plastic flasks. The cultures are incubated at 37°C with vigorous aeration (approximately 200 rpm) for four to five days. Culture broths are harvested by
25 centrifugation and the amount of laccase activity in the supernatant is determined using syringaldazine as a substrate. Briefly, 800 µl of assay buffer (25 mM sodium acetate, pH 5.5, 40 µM CuSO₄) is mixed with 20 µl of culture supernatant and 60
30 µl of 0.28 mM syringaldazine stock solution (Sigma Chemical Co., St. Louis, MO) in 50% ethanol. The absorbance at 530 nm is measured over time in a Genesys 5 UV-vis spectrophotometer (Milton-Roy). One laccase unit (LACU) is defined as the amount of enzyme which oxidizes one µmole of substrate per minute at room temperature. SDS-polyacrylamide gel

electrophoresis(PAGE) is done using precast 10-27% gradient gels from Novex(San Diego, CA). Protein bands are developed using Coomassie Brilliant Blue(Sigma).

5 B.RESULTS AND DISCUSSION

1. Expression of *Scytalidium* laccase

The expression vector pShTh15 is used in conjunction with pPyrG (*A. nidulans* pyrG) or pToC90(*A. nidulans* amdS) plasmids to generate *A. oryzae* and *A. niger* co-transformants which
10 express StL. As shown in Table 1, the number of laccase-producing co-transformants obtained in *A. oryzae* HowB104pyrG is small (3.7% of Pyr+ transformants) compared to the number obtained in *A. niger* Bo-1 using amdS selection (71.5% of AmdS+ transformants). It is unknown whether this is due to an
15 abnormally low co-transformation(i.e., integration) frequency or extremely low expression or laccase degradation in many *A. oryzae* transformants. Expression levels of StL range from about 50mg/l in shake flasks and 1-2g/l in a fermentor.

20

III. PURIFICATION AND CHARACTERIZATION OF RECOMBINANT SCYTALIDIUM LACCASE

A. MATERIALS AND METHODS

1. Materials

25 Chemicals used as buffers and substrates are commercial products of at least reagent grade. Chromatography is performed on either a Pharmacia FPLC. Spectroscopic assays are conducted on either a spectrophotometer(Shimadzu PC160) or a microplate reader(Molecular Devices). Britton &
30 Robinson(B&R) buffers are prepared according to the protocol described in Quelle, Biochemisches Taschenbuch, H.M. Raven, II. Teil, S.93 u. 102, 1964.

2. Fermentation

A 1 ml aliquot of a spore suspension of *Aspergillus oryzae* transformant HowB104-pShTh15-2 (approximately 10^9 spores/ml) is added aseptically to a 500 ml shake flask containing 100 ml of sterile shake flask medium (maltose, 50g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2g/l; KH_2PO_4 , 10g/l; K_2SO_4 , 2g/l; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.5 g/l; Citric acid, 2g/l; yeast extract, 10g/l; trace metals [$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 14.3 g/l; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2.5 g/l; $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 g/l; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 13.8 g/l; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 8.5 g/l; citric acid, 3.0 g/l], 0.5 ml/l; urea, 2g/l, made with tap water and adjusted to pH 6.0 before autoclaving), and incubated at 37°C on a rotary shaker at 200 rpm for 18 hours. 50 ml of this culture is aseptically transferred to a 3 liter fermentor containing 1.8 liters of the fermentor media ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2g/l; KH_2PO_4 , 2g/l; citric acid 4g/l; K_2SO_4 , 3g/l; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 2g/l; trace metals, 0.5 ml/l; pluronic antifoam, 1ml/l). The fermentor temperature is maintained at 34°C by the circulation of cooling water through the fermentor jacket. Sterile air is sparged through the fermentor at a rate of 1.8 liter/min (1v/v/m). The agitation rate is maintained between 600 and 1300 rpm at approximately the minimum level required to maintain the dissolved oxygen level in the culture above 20%. Sterile feed (Nutriose 725 [maltose syrup], 225 g/l; urea, 30 g/l; yeast extract, 15 g/l; pluronic antifoam, 1.5 ml/l, made up with distilled water and autoclaved) is added to the fermentor by use of a peristaltic pump. The feed rate profile during the fermentation is as follows: 30 g of feed is added initially before inoculation; 0-24 h, 2 g/l h; 24-48 h, 4 g/l h; 48h-end, 6 g/l.

Copper (in the form of CuCl_2 , CuSO_4 or other soluble salt) is made as a 400X stock in water or a suitable buffer, filter sterilized and added aseptically to the tank to a final level of 0.5 mM.

Samples for enzyme activity determination are withdrawn and filtered through Miracloth to remove mycelia. These samples are assayed for laccase activity by the LACU assay described above. Laccase activity is found to increase continuously during the course of the fermentation, with a value of approximately 3.6 LACU/ml achieved after 115 hours in the fermentation containing excess copper. At a specific activity of 1.9 LACU/mg, this corresponds to over 1.8 g/l recombinant laccase expressed by this transformant.

10 3. Enzymatic Assay

Laccase activity is determined by syringaldazine oxidation at 30°C in a 1-cm quartz cuvette. 60µl syringaldazine stock solution (0.28 mM in 50% ethanol) and 20 µl sample are mixed with 0.8 ml preheated buffer solution. The oxidation is monitored at 530nm over 5 minutes. The activity is expressed as µmole substrate oxidized per minute. B&R buffers with various pHs are used. The activity unit is referred to here as "SOU". A buffer of 25 mM sodium acetate, 40 µM CuSO₄, pH 5.5, is also used to determine the activity, which is referred to as LACU, as defined above. 2,2'-azinobis(3-ethylbenzo thiazoline-6-sulfonic acid) (ABTS) oxidation assays are done using 0.4 mM ABTS, B&R buffer, pH 4.1, at room temperature by monitoring ΔA₄₀₅. An ABTS oxidase activity overlay assay is performed by pouring cooled ABTS-agarose (0.05 g ABTS, 1 g agarose, 50 ml H₂O, heated to dissolve agarose) over a native-IEF gel and incubating at room temperature. Thermostability analysis is performed using samples that have ~3 µM enzyme preincubated for one hour in B&R buffer, at pH 2.7, 6.1, and 9.0, and various temperatures. Samples are assayed after a 44-fold dilution into B & R buffer, pH 4.1, at room temperature.

30 3. Purification from a fermentor broth

1.2 liters of cheese-cloth filtered broth (pH 7.9, 13 mS) is filtered through Whatman #2 filter paper and concentrated

on a Spiral Concentrator (Amicon) with a S1Y100 membrane (MWCO:100) to 200 ml. The concentrate is adjusted to 0.86 mS by diluting it in water and reconcentrated on S1Y100 to 324 ml. The washed and concentrated broth has a dense greenish
5 color.

The broth is frozen overnight at -20°C, thawed the next day (without any loss of activity) and loaded onto a Q-Sepharose XK26 column (120 ml), preequilibrated with 10 mM Tris, pH 7.7, 0.9 mS. The blue laccase band is eluted during
10 a linear gradient with 2 M NaCl.

Pooled laccase fractions (44 ml), dialyzed in 3.5 liters of 10 mM NaAc, pH 5.5, 0.8 mS at 4°C overnight, are loaded onto a Mono-Q 16/10 (40 ml), preequilibrated with 10 mM MES, pH 5.3, 0.8 mS. The laccase eluted during a linear gradient
15 with 1 M NaCl shows apparent homogeneity on SDS-PAGE.

4. Analysis of amino acid content and N-terminus

N-terminal sequencing is performed on an ABI 476A sequencer; and total amino acid analysis, from which the extinction coefficient of laccase is determined, is performed
20 on a HP AminoQuant instrument.

B. RESULTS AND DISCUSSION

1. Purification

From 1200 ml fermentor broth, about 0.6g of laccase are
25 isolated. Initial concentration using a membrane with MWCO of 100 kDa removes significant amounts of brown material and small contaminant proteins. The low affinity of the laccase toward Q-Sepharose matrix equilibrated with 10 mM Tris, pH 7.7, facilitates its separation from other impurities. The
30 enriched fractions are further purified by Mono-Q at pH 5.3. Although it has a pI of 5.1, the laccase migrates slowly on Mono-Q and is separated from impurities during the washing by 10 mM MES, pH 5.3. An overall 15-fold purification and a recovery of 60% are achieved.

2. Characterization

The purified laccase shows a MW of 75-80 kDa on SDS-PAGE. The difference between the MW derived from DNA sequence (63 kDa) and the observed MW is attributable to glycosylation.

- 5 Native IEF shows 3 bands near pI of about 5.1, which are active in ABTS overlay assay.

3. N-terminal sequencing

- Directly sequencing the N-terminus of the purified laccase from samples either in desalted solution or on PVDF
10 membrane are unsuccessful. This result suggests a blocked N-terminus, likely a pyroglutamate site based on the gene sequence.

- The spectrum of the blue laccase has absorption maxima at 276 and 602 nm; with $Abs_{280}/Abs_{600}=23$ and $Abs_{330}/Abs_{589}=2.1$. The
15 extinction coefficient determined by amino acid analysis is 1.9 l/(g*cm).

- The activity is tested by using either syringaldazine or ABTS as substrates. Expressed as per Abs_{280} or per mg, the laccase has a value of 2.2 or 4.2 units for SOU at pH 7,
20 respectively.

- The pH profiles of laccase activity has optimal pH of 7 and 4, for syringaldazine and ABTS oxidation, respectively (Figure 4). Thermostability analysis at three pHs is shown in Figure 5. The laccase is more stable at neutral
25 to alkaline pH than at acidic pH. Thermoactivation is also observed in neutral-alkaline pH range.

Deposit of Biological Materials

- 30 The following biological material has been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria, Illinois, 61604 and given the following accession number.

Deposit

E. coli JM101 containing
pShTh15

5

Accession Number

NRRL B-21262

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Novo Nordisk Biotech, Inc.
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(ii) TITLE OF INVENTION: PURIFIED SCYTALIDIUM LACCASES AND NUCLEIC ACIDS ENCODING SAME

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:

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- (F) ZIP: 10174-6401

(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: US
- (B) FILING DATE: 31-May-95
- (C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/253,784
- (B) FILING DATE: 03-June-1994

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2476 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Scytalidium thermophilum

(ix) FEATURE:

- (A) NAME/KEY: intron

(B) LOCATION: 349..411

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 502..559

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 632..686

(ix) FEATURE:

(A) NAME/KEY: intron

(B) LOCATION: 1739..1804

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: join (106..348, 412..501, 560..631, 687..1738,
1805..2194)

(xi). SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CTGAATTTAA ATACAGGAAG ATCGCATTCA ATCCAGCCTA GACTGCACAA TGGTTCTGCA	60
CGACCGTCGC ACACCTGCCA ATAGTGTTAA TAACGGNCTA ATACC ATG AAG CGC TTC	117
Met Lys Arg Phe	1
TTC ATT AAT AGC CTT CTG CTT CTC GCA GGG CTC CTC AAC TCA GGG GCC	165
Phe Ile Asn Ser Leu Leu Leu Leu Ala Gly Leu Leu Asn Ser Gly Ala	5 10 15 20
CTC GCG GCT CCG TCT ACA CAT CCC AGA TCA AAC CCC GAC ATA CTG CTT	213
Leu Ala Ala Pro Ser Thr His Pro Arg Ser Asn Pro Asp Ile Leu Leu	25 30 35
GAA AGA GAT GAC CAC TCC CTT ACG TCT CGG CAA GGT AGC TGT CAT TCT	261
Glu Arg Asp Asp His Ser Leu Thr Ser Arg Gln Gly Ser Cys His Ser	40 45 50
CCA AGC AAC CGC GCC TGT TGG TGC TCT GGC TTC GAT ATC AAC ACG GAT	309
Pro Ser Asn Arg Ala Cys Trp Cys Ser Gly Phe Asp Ile Asn Thr Asp	55 60 65
TAT GAG ACC AAG ACT CCA AAC ACC GGA GTG GTG CGG CGG GTTAGTATCC	358
Tyr Glu Thr Lys Thr Pro Asn Thr Gly Val Val Arg Arg	70 75 80
CAAGTTACGT TTGACCAAGA AATGGACGTG AAGTGTGCTG ACTCTCCCGC TAG	411
TAC ACC TTT GAT ATC ACC GAA GTC GAC AAC CGC CCC GGT CCC GAT GGG	459
Tyr Thr Phe Asp Ile Thr Glu Val Asp Asn Arg Pro Gly Pro Asp Gly	85 90 95
GTC ATC AAG GAG AAG CTC ATG CTT ATC AAC GAC AAA CTC CTG GTAGG	506
Val Ile Lys Glu Lys Leu Met Leu Ile Asn Asp Lys Leu Leu	100 105 110
GTCCTCTCGA ACGCCTGCGT CTGCCACACA GCGTAAACT AACGAACCGC TAG	559
GGC CCG ACA GTC TTC GCA AAC TGG GGC GAC ACC ATC GAG GTG ACC GTC	607
Gly Pro Thr Val Phe Ala Asn Trp Gly Asp Thr Ile Glu Val Thr Val	115 120 125
AAC AAC CAC CTG AGA ACC AAC GGA GTAAGCGTTC GGACACAAAG CCCAGCAACC	661
Asn Asn His Leu Arg Thr Asn Gly	130 135

TAGACACACT	CAACTGACCA	AGTAG	ACC	TCC	ATC	CAC	TGG	CAC	GGC	TTG	CAC	CAA	716
			Thr	Ser	Ile	His	Trp	His	Gly	Leu	His	Gln	
							140					145	
AAA GGA ACC AAC TAC CAC GAC GGC GCC AAC GGC GTG ACC GAG TGT CCC													764
Lys Gly Thr Asn Tyr His Asp Gly Ala Asn Gly Val Thr Glu Cys Pro													
			150				155					160	
ATC CCG CCC GGT GGC TCC CGA GTC TAC AGC TTC CGA GCG CGC CAA TAT													812
Ile Pro Pro Gly Gly Ser Arg Val Tyr Ser Phe Arg Ala Arg Gln Tyr													
			165				170				175		
GGA ACG TCA TGG TAC CAC TCC CAC TTC TCC GCC CAG TAT GGC AAC GGC													860
Gly Thr Ser Trp Tyr His Ser His Phe Ser Ala Gln Tyr Gly Asn Gly													
			180				185				190		
GTG AGC GGC GCC ATC CAG ATC AAC GGA CCC GCC TCC CTG CCC TAC GAC													908
Val Ser Gly Ala Ile Gln Ile Asn Gly Pro Ala Ser Leu Pro Tyr Asp													
			195			200			205				
ATC GAC CTC GGC GTC CTC CCG CTG CAG GAC TGG TAC TAC AAG TCC GCC													956
Ile Asp Leu Gly Val Leu Pro Leu Xaa Asp Trp Tyr Tyr Lys Ser Ala													
			210			215			220				225
GAC CAG CTC GTC ATC GAG ACC CTG GCC AAG GGC AAC GCT CCG TTC AGC													1004
Asp Gln Leu Val Ile Glu Thr Leu Xaa Lys Gly Asn Ala Pro Phe Ser													
			230				235						240
GAC AAC GTC CTC ATC AAC GGC ACC GCA AAG CAC CCC ACC ACT GGC GAA													1052
Asp Asn Val Leu Ile Asn Gly Thr Ala Lys His Pro Thr Thr Gly Glu													
			245				250					255	
GGG GAG TAC GCC ATC GTG AAG CTC ACC CCG GGC AAA CGC CAT CGC CTG													1100
Gly Glu Tyr Ala Ile Val Lys Leu Thr Pro Asp Lys Arg His Arg Leu													
			260			265					270		
CGG CTC ATC AAC ATG TCG GTG GAG AAC CAC TTC CAG GTC TCG CTG GCG													1148
Arg Leu Ile Asn Met Ser Val Glu Asn His Phe Gln Val Ser Leu Ala													
			275			280			285				
AAG CAC ACC ATG ACG GTC ATC GCG GCG GAC ATG GTC CCC GTC AAC GCC													1196
Lys His Thr Met Thr Val Ile Ala Ala Asp Met Val Pro Val Asn Ala													
			290			295			300				305
ATG ACC GTC GAC AGC CTG TTT ATG GCC GNC GGG CAG CGG TAT GAT GTT													1244
Met Thr Val Asp Ser Leu Phe Met Ala Val Gly Gln Arg Tyr Asp Val													
			310				315					320	
ACC ATC GAC GCG AGC CAG GCG GTG GGG AAT TAC TGG TTC AAC ATC ACC													1292
Thr Ile Asp Ala Ser Gln Ala Val Gly Asn Tyr Trp Phe Asn Ile Thr													
			325				330				335		
TTT GGA GGG CAG CAG AAG TGC GGC TTC TCG CAC AAT CCG GCG CCG GCA													1340
Phe Gly Gly Gln Gln Lys Cys Gly Phe Ser His Asn Pro Ala Pro Ala													
			340			345					350		
GCC ATC TTT CCG TAC GAG GGC GCT CCT GAC GCT CTG CCG ACG GAT CCT													1388
Ala Ile Phe Arg Tyr Glu Gly Ala Pro Asp Ala Leu Pro Thr Asp Pro													
			355			360			365				
GGC GCT GCG CCA AAG GAT CAT CAG TGC CTG GAC ACT TTG GAT CTT TCA													1436
Gly Ala Ala Pro Lys Asp His Gln Cys Leu Asp Thr Leu Asp Leu Ser													
			370			375			380				385
CCG GTG GTG CAA AAG AAC GTG CCG GTT GAC GGG TTC GTC AAA GAG CCT													1484
Pro Val Val Gln Lys Asn Val Pro Val Asp Gly Phe Val Lys Glu Pro													
			390				395					400	

GGC AAT ACG CTG CCG GTG ACG CTC CAT GTT GAC CAG GCC GCG GCT CCA Gly Asn Thr Leu Pro Val Thr Leu His Val Asp Gln Ala Ala Ala Pro 405 410 415	1532
CAC GTG TTT ACG TGG AAG ATC AAC GGG AGC GCT GCG GAC GTG GAC TGG His Val Phe Thr Trp Lys Ile Asn Gly Ser Ala Ala Asp Val Asp Trp 420 425 430	1580
GAC AGG CCG GTG CTG GAG TAT GTC ATG AAC AAT GAC CTG TCT AGC AAT Asp Arg Pro Val Leu Glu Tyr Val Met Asn Asn Asp Leu Ser Ser Ile 435 440 445	1628
CCG GTC AAG AAC AAC ATT GTG AGG GTG GAC GGA GTC AAC GAG TGG ACG Pro Val Lys Asn Asn Ile Val Arg Val Asp Gly Val Asn Glu Trp Thr 450 455 460 465	1676
TAC TGG CTC GTC GAA AAC GAC CCG GAG GGC CGC CTC AGT TTG CCG CAT Tyr Trp Leu Val Glu Asn Asp Pro Glu Gly Arg Leu Ser Leu Pro His 470 475 470	1724
CCG ATG CAT CTA CAC GTAAGTCACA TCCCCACTA CCATTCGGAA TGACCACCAG Pro Met His Leu His 475	1779
GTACTGACAC CCTCCTCCTC AATAG GGA CAC GAT TTC TTT GTC CTA GGC CGC Gly His Asp Phe Phe Val Leu Gly Arg 480 485	1831
TCC CCC GAC GTC TCG CCC GAT TCA GAA ACC CGC TTC GTC TTT GAC CCG Ser Pro Asp Val Ser Pro Asp Ser Glu Thr Arg Phe Val Phe Asp Pro 490 495 500	1879
GCC GTC GAC CTC CCC CGT CTG CGC GGA CAC AAC CCC GTC CGG CGC GAC Ala Val Asp Leu Pro Arg Leu Arg Gly His Asn Pro Val Arg Arg Asp 505 510 515	1927
GTC ACC ATG CTT CCC GCG CGC GGC TGG CTG CTG CTG GCC TTC CGC ACG Val Thr Met Leu Pro Ala Arg Glu Trp Leu Leu Leu Ala Phe Arg Thr 520 525 530	1975
GAC AAC CCG GGC GCG TGG TTG TTC CAC TGC CAC ATC GCG TGR CAC GTG Asp Asn Pro Gly Ala Trp Phe His Cys His Ile Ala Trp His Val 535 540 545	2023
TCG GGC GGG TTA AGC GTC GAC TTT CTG GAG CGG CCG GAC GAG CTG CGC Ser Gly Gly Leu Ser Val Asp Phe Leu Glu Arg Pro Asp Glu Leu Arg 550 555 560 565	2071
GGG CAG CTG ACG GGA GAG AGC AAG GCG GAG TTC GAG CGT GTT TGT CGC Gly Gln Leu Thr Gly Glu Ser Lys Ala Glu Leu Glu Arg Val Cys Arg 570 575 580	2119
GAG TGG AAG GAT TGG GAG CCG AAG AGC CCG CAT GGG AAG ATC GAT TCG Glu Trp Lys Asp Trp Glu Ala Lys Ser Pro His Gly Lys Ile Asp Ser 585 590 595	2167
GGG TTG AAG CAG CGG CGA TGG GAT GCG TGAGGTAGTT GGGCGGATTG Gly Leu Lys Gln Arg Arg Trp Asp Ala 600 605	2214
TTTAACACGT AGTGGGTAAG GTTGGGGCGG GTTTGTTTGG CGTTTTTCAGG GGTGGGGGTG	2274
CGGATGCTGG TCATCCGGGA AACGGCTCTA CAACTGGTGT CAATAGACTA ATATAGAGTG	2334
ATCAAAGAAC TGAGGTTCTG AAAGAGGCGT GGAAGTCGCG TTGTGACTCC CTTTGCCATG	2394
TTGGGAAGTG TGGCTCAACA TTGTGTTTCAG GTTTGCTCAG GGTGATNTCG AACTGACGTN	2454

TTGATGAGGG TTATTGCNTA GA

2476

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 616 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Scytalidium thermophilum*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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Met Lys Arg Phe Phe Ile Asn Ser Leu Leu Leu Leu Ala Gly Leu Leu
 1           5           10           15

Asn Ser Gly Ala Leu Ala Ala Pro Ser Thr His Pro Arg Ser Asn Pro
 20           25           30

Asp Ile Leu Leu Glu Arg Asp Asp His Ser Leu Thr Ser Arg Gln Gly
 35           40           45

Ser Cys His Ser Pro Ser Asn Arg Ala Cys Trp Cys Ser Gly Phe Asp
 50           55           60

Ile Asn Thr Asp Tyr Glu Thr Lys Thr Pro Asn Thr Gly Val Val Arg
 65           70           75           80

Arg Tyr Thr Phe Asp Ile Thr Glu Val Asp Asn Arg Pro Gly Pro Asp
 85           90           95

Gly Val Ile Lys Glu Lys Leu Met Leu Ile Asn Asp Lys Leu Leu Gly
100           105           110

Pro Thr Val Phe Ala Asn Trp Gly Asp Thr Ile Glu Val Thr Val Asn
115           120           125

Asn His Leu Arg Thr Asn Gly Thr Ser Ile His Trp His Gly Leu His
130           135           140

Gln Lys Gly Thr Asn Tyr His Asp Gly Ala Asn Gly Val Thr Glu Cys
145           150           155           160

Pro Ile Pro Pro Gly Gly Ser Arg Val Tyr Ser Phe Arg Ala Arg Gln
165           170           175

Tyr Gly Thr Ser Trp Tyr His Ser His Phe Ser Ala Gln Tyr Gly Asn
180           185           190

Gly Val Ser Gly Ala Ile Gln Ile Asn Gly Pro Ala Ser Leu Pro Tyr
195           200           205

Asp Ile Asp Leu Gly Val Leu Pro Leu Gln Asp Trp Tyr Tyr Lys Ser
210           215           220

Ala Asp Gln Leu Val Ile Glu Thr Leu Ala Lys Gly Asn Ala Pro Phe
225           230           235           240

Ser Asp Asn Val Leu Ile Asn Gly Thr Ala Lys His Pro Thr Thr Gly
245           250           255

Glu Gly Glu Tyr Ala Ile Val Lys Leu Thr Pro Asp Lys Arg His Arg
260           265           270

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Leu Arg Leu Ile Asn Met Ser Val Glu Asn His Phe Gln Val Ser Leu
 275 280 285
 Ala Lys His Thr Met Thr Val Ile Ala Ala Asp Met Val Pro Val Asn
 290 295 300
 Ala Met Thr Val Asp Ser Leu Phe Met Ala Xaa Gly Gln Arg Tyr Asp
 305 310 315 320
 Val Thr Ile Asp Ala Ser Gln Ala Val Gly Asn Tyr Trp Phe Asn Ile
 325 330 335
 Thr Phe Gly Gly Gln Gln Lys Cys Gly Phe Ser His Asn Pro Ala Pro
 340 345 350
 Ala Ala Ile Phe Arg Tyr Glu Gly Ala Pro Asp Ala Leu Pro Thr Asp
 355 360 365
 Pro Gly Ala Ala Pro Lys Asp His Gln Cys Leu Asp Thr Leu Asp Leu
 370 375 380
 Ser Pro Val Val Gln Lys Asn Val Pro Val Asp Gly Phe Val Lys Glu
 385 390 395 400
 Pro Gly Asn Thr Leu Pro Val Thr Leu His Val Asp Gln Ala Ala Ala
 405 410 415
 Pro His Val Phe Thr Trp Lys Ile Asn Gly Ser Ala Ala Asp Val Asp
 420 425 430
 Trp Asp Arg Pro Val Leu Glu Tyr Val Met Asn Asn Asp Leu Ser Ser
 435 440 445
 Ile Pro Val Lys Asn Asn Ile Val Arg Val Asp Gly Val Asn Glu Trp
 450 455 460
 Thr Tyr Trp Leu Val Glu Asn Asp Pro Glu Gly Arg Leu Ser Leu Pro
 465 470 475 480
 His Pro Met His Leu His Gly His Asp Phe Phe Val Leu Gly Arg Ser
 485 490 495
 Pro Asp Val Ser Pro Asp Ser Glu Thr Arg Phe Val Phe Asp Pro Ala
 500 505 510
 Val Asp Leu Pro Arg Leu Arg Gly His Asn Pro Val Arg Arg Asp Val
 515 520 525
 Thr Met Leu Pro Ala Arg Gly Trp Leu Leu Leu Ala Phe Arg Thr Asp
 530 535 540
 Asn Pro Gly Ala Trp Leu Phe His Cys His Ile Ala Trp His Val Ser
 545 550 555 560
 Gly Gly Leu Ser Val Asp Phe Leu Glu Arg Pro Asp Glu Leu Arg Gly
 565 570 575
 Gln Leu Thr Gly Glu Ser Lys Ala Glu Leu Glu Arg Val Cys Arg Glu
 580 585 590
 Trp Lys Asp Trp Glu Ala Lys Ser Pro His Gly Lys Ile Asp Ser Gly
 595 600 605
 Leu Lys Gln Arg Arg Trp Asp Ala
 610 615

Applicant's or agent's file reference number	4186.204-WO	International application N	TBA
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13 bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>29</u> , line <u>3</u>	
B. IDENTIFICATION OF Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depository institution Agricultural Research Service Patent Culture Collection (NRRL)	
Address of depository institution (including postal code and country) Northern Regional Research Center 1815 University Street Peoria, IL 61604, US	
Date of deposit May 25, 1995	Accession Number NRRL B-21262
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European and/or Australia Patent is sought, during the pendency of the patent application, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (Rule 28(4) EPC/Regulation 3.25 of Australia Statutory Rule 1991 No. 71).	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indication listed below will be submitted to the International Bureau Later (specify the general nature of the indications e.g. "Accession Number of Deposit")	

For receiving Office use only

For International Bureau use only

<input checked="" type="checkbox"/> This sheet was received with the international application
Authorized officer Doris L. Erick <i>DL</i> PCT International Division

<input type="checkbox"/> This sheet was received with the International Bureau on:
Authorized officer

What we claim is:

1. A DNA construct containing a sequence encoding a *Scytalidium* laccase.
- 5 2. The construct of Claim 1 which comprises a sequence encoding a *Scytalidium thermophilum* laccase.
3. The construct of Claim 1 which comprises a sequence
10 encoding the amino acid sequence depicted in SEQ ID NO. 2.
4. The construct of Claim 1, which comprises the nucleic acid sequence depicted in SEQ ID NO. 1.
- 15 5. The construct of Claim 1, which comprises the nucleic acid sequence contained in NRRL B-21262.
6. A substantially pure *Scytalidium* laccase enzyme.
- 20 7. The enzyme of Claim 6 which is a *Scytalidium thermophilum* laccase.
8. The enzyme of Claim 6 which comprises the sequence depicted in SEQ ID NO. 2, or a sequence with at least about
25 80% homology thereto.
9. A recombinant vector comprising a DNA construct containing a sequence encoding a *Scytalidium* laccase.
- 30 10. The vector of Claim 9 in which the sequence is operably linked to a promoter sequence.
11. The vector of Claim 10 in which the promoter is a fungal or yeast promoter.

12. The vector of Claim 11 in which the promoter is the TAKA amylase promoter of *Aspergillus oryzae*.
- 5 13. The vector of Claim 11 in which the promoter is the glucoamylase (*glaA*;) promoter of *Aspergillus niger* or *Aspergillus awamori*.
14. The vector of Claim 9 which also comprises a selectable
10 marker.
15. The vector of Claim 14 in which the selectable marker is selected from the group consisting of *amdS*, *pyrG*, *argB*, *niaD*, *sC*, and *hygB*.
- 15 16. The vector of Claim 14 in which the selectable marker is the *amdS* marker of *Aspergillus nidulans* or *Aspergillus oryzae*, or the *pyrG* marker of *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus awamori*, or *Aspergillus oryzae*.
- 20 17. The vector of Claim 14 which comprises both the TAKA amylase promoter of *Aspergillus oryzae* and the *amdS* or *pyrG* marker of *Aspergillus nidulans* or *Aspergillus oryzae*.
- 25 18. A recombinant host cell comprising a heterologous DNA construct containing a nucleic acid sequence encoding a *Scytalidium laccase*.
19. The host cell of Claim 18 which is a fungal cell.
- 30 20. The host cell of Claim 19 which is an *Aspergillus* cell.
21. The host cell of Claim 18 in which the construct is integrated into the host cell genome.

22. The host cell of Claim 18 in which the construct is contained on a vector.
- 5 23. The host cell of Claim 18 which comprises a construct containing a sequence encoding the amino acid sequence depicted in SEQ ID NO. 2.
24. A method for obtaining a laccase enzyme which comprises
10 culturing a host cell comprising a DNA construct containing a sequence encoding a *Scytalidium* laccase enzyme, under conditions conducive to expression of the enzyme, and recovering the enzyme from the culture.
- 15 25. A *Scytalidium* enzyme obtained by the method of Claim 24.
26. A method for polymerizing a lignin or lignosulfate substrate in solution which comprises contacting the substrate with a *Scytalidium* laccase.
- 20 27. A method for in situ depolymerization in Kraft pulp which comprises contacting the pulp with a *Scytalidium* laccase.
28. A method for oxidizing dyes or dye precursors which
25 comprises contacting the dye or dye precursor with a *Scytalidium* laccase.
29. A method of polymerizing or oxidizing a phenolic compound which comprises contacting the phenolic compound with a
30 *Scytalidium* laccase.

1	CTGAATTTAAATACAGGAAGATCGCATTCAATCCAGCCTAGACTGCACAATGGTTCTGCA	60
1		1
61	CGACCGTCGCACACCTGCCAATAGTGTTAATAACGGCCTAATACC ATG AAG CGC TT	116
1		4
		M K R F
117	C TTC ATT AAT AGC CTT CTG CTT CTC GCA GGG CTC CTC AAC TCA GG	161
4	F I N S L L L L A G L L N S G	19
162	G GCC CTC GCG GCT CCG TCT ACA CAT CCC AGA TCA AAC CCC GAC AT	206
19	A L A A P S T H P R S N P D I	34
207	A CTG CTT GAA AGA GAT GAC CAC TCC CTT ACG TCT CGG CAA GGT AG	251
34	L L E R D D H S L T S R Q G S	49
252	C TGT CAT TCT CCA AGC AAC CGC GCC TGT TGG TGC TCT GGC TTC GA	296
49	C H S P S N R A C W C S G F D	64
297	T ATC AAC ACG GAT TAT GAG ACC AAG ACT CCA AAC ACC GGA GTG GT	341
64	I N T D Y E T K T P N T G V V	79
342	G CGG CGG GTTAGTATCCCAAGTTACGTTTGACCAAGAAATGGACGTGAAGTGTGCTG	398
79	R R	81
399	ACTCTCCCGCTAG TAC ACC TTT GAT ATC ACC GAA GTC GAC AAC CGC CC	446
81		12
	Y T F D I T E V D N R P	
447	C GGT CCC GAT GGG GTC ATC AAG GAG AAG CTC ATG CTT ATC AAC GA	491
12	G P D G V I K E K L M L I N D	27
492	C AAA CTC CTG G GTAGGTCCTCTCGAACGCCTGCGTCTGCCACACAGCGTAAACT	547
27	K L L	31
548	AACGAACCGCTAG GC CCG ACA GTC TTC GCA AAC TGG GGC GAC ACC ATC	595
31		11
	P T V F A N W G D T I	
596	GAG GTG ACC GTC AAC AAC CAC CTG AGA ACC AAC GG GTAAGCGTTCGGA	643
11	E V T V N N H L R T N	23
644	CACAAAGCCCAGCAACCTAGACACACTCAACTGACCAAGTAG A ACC TCC ATC CAC	698
23		4
	T S I H	

FIG. 1A

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699	TGG CAC GGC TTG CAC CAA AAA GGA ACC AAC TAC CAC GAC GGC GCC	743
4	W H G L H Q K G T N Y H D G A	19
744	AAC GGC GTG ACC GAG TGT CCC ATC CCG CCC GGT GGC TCC CGA GTC	788
19	N G V T E C P I P P G G S R V	34
789	TAC AGC TTC CGA GCG CGC CAA TAT GGA ACG TCA TGG TAC CAC TCC	833
34	Y S F R A R Q Y G T S W Y H S	49
834	CAC TTC TCC GCC CAG TAT GGC AAC GGC GTG AGC GGC GCC ATC CAG	878
49	H F S A Q Y G N G V S G A I Q	64
879	ATC AAC GGA CCC GCC TCC CTG CCC TAC GAC ATC GAC CTC GGC GTC	923
64	I N G P A S L P Y D I D L G V	79
924	CTC CCG CTG CAG GAC TGG TAC TAC AAG TCC GCC GAC CAG CTC GTC	968
79	L P L Q D W Y Y K S A D Q L V	94
969	ATC GAG ACC CTG GCC AAG GGC AAC GCT CCG TTC AGC GAC AAC GTC	1013
94	I E T L A K G N A P F S D N V	109
1014	CTC ATC AAC GGC ACC GCA AAG CAC CCC ACC ACT GGC GAA GGG GAG	1058
109	L I N G T A K H P T T G E G E	124
1059	TAC GCC ATC GTG AAG CTC ACC CCG GGC AAA CCG CAT CGC CTG CCG	1103
124	Y A I V K L T P G K R H R L R	139
1104	CTC ATC AAC ATG TCG GTG GAG AAC CAC TTC CAG GTC TCG CTG GCG	1148
139	L I N M S V E N H F Q V S L A	154
1149	AAG CAC ACC ATG ACG GTC ATC GCG GCG GAC ATG GTC CCC GTC AAC	1193
154	K H T M T V I A A D M V P V N	169
1194	GCC ATG ACC GTC GAC AGC CTG TTT ATG GCC GNC GGG CAG CGG TAT	1238
169	A M T V D S L F M A X G Q R Y	184
1239	GAT GTT ACC ATC GAC GCG AGC CAG GCG GTG GGG AAT TAC TGG TTC	1283
184	D V T I D A S Q A V G N Y W F	199
1284	AAC ATC ACC TTT GGA GGG CAG CAG AAG TGC GGC TTC TCG CAC AAT	1328
199	N I T F G G Q Q K C G F S H N	214

FIG.1B

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SUBSTITUTE SHEET (RULE 26)

1329	CCG GCG CCG GCA GCC ATC TTT CGC TAC GAG GGC GCT CCT GAC GCT	1373
214	P A P A A I F R Y E G A P D A	229
1374	CTG CCG ACG GAT CCT GGC GCT GCG CCA AAG GAT CAT CAG TGC CTG	1418
229	L P T D P G A A P K D H Q C L	244
1419	GAC ACT TTG GAT CTT TCA CCG GTG GTG CAA AAG AAC GTG CCG GTT	1463
244	D T L D L S P V V Q K N V P V	259
1464	GAC GGG TTC GTC AAA GAG CCT GGC AAT ACG CTG CCG GTG ACG CTC	1508
259	D G F V K E P G N T L P V T L	274
1509	CAT GTT GAC CAG GCC GCG GCT CCA CAC GTG TTT ACG TGG AAG ATC	1553
274	H V D Q A A A P H V F T W K I	289
1554	AAC GGG AGC GCT GCG GAC GTG GAC TGG GAC AGG CCG GTG CTG GAG	1598
289	N G S A A D V D W D R P V L E	304
1599	TAT GTC ATG AAC AAT GAC CTG TCT AGC ATT CCG GTC AAG AAC AAC	1643
304	Y V M N N D L S S I P V K N N	319
1644	ATT GTG AGG GTG GAC GGA GTC AAC GAG TGG ACG TAC TGG CTC GTC	1688
319	I V R V D G V N E W T Y W L V	334
1689	GAA AAC GAC CCG GAG GGC CGC CTC AGT TTG CCG CAT CCG ATG CAT	1733
334	E N D P E G R L S L P H P M H	349
1734	CTA CAC GTAAGTCACATCCCCCACTACCATTCCGAATGACCACCAGGTACTGACACC	1790
349	L H	351
1791	CTCCTCCTCAATAG GGA CAC GAT TTC TTT GTC CTA GGC CGC TCC CCC G	1838
351	G H D F F V L G R S P	12
1839	AC GTC TCG CCC GAT TCA GAA ACC CGC TTC GTC TTT GAC CCG GCC G	1883
12	D V S P D S E T R F V F D P A	27
1884	TC GAC CTC CCC CGT CTG CGC GGA CAC AAC CCC GTC CCG CGC GAC G	1928
27	V D L P R L R G H N P V R R D	42
1929	TC ACC ATG CTT CCC GCG CGC GGC TGG CTG CTG CTG GCC TTC CGC A	1973
42	V T M L P A R G W L L L A F R	57

FIG.1C

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1974	CG GAC AAC CCG GGC GCG TGG TTG TTC CAC TGC CAC ATC GCG TGR C	2018
57	T D N P G A W L F H C H I A X	72
2019	AC GTG TCG GGC GGG TTA AGC GTC GAC TTT CTG GAG CCG CCG GAC G	2063
72	H V S G G L S V D F L E R P D	87
2064	AG CTG CCG GGC CAG CTG ACG GGA GAG AGC AAG GCG GAG TTG GAG C	2108
87	E L R G Q L T G E S K A E L E	102
2109	GT GTT TGT CCG GAG TGG AAG GAT TGG GAG GCG AAG AGC CCG CAT G	2153
102	R V C R E W K D W E A K S P H	117
2154	GG AAG ATC GAT TCG GGC TTG AAG CAG CCG CGA TGG GAT GCG TGA G	2198
117	G K I D S G L K Q R R W D A *	131
2199	GTAGTTGGCGCGATTGTTTAACACGTAGTGGTAAGGTTGGGCGGGTTTGTTCGCGTT	2258
131		131
2259	TTCAGGGGTTGGGTCGGGATGCTGGTCATCGGGAACGGCTCTACAACGGTGTCAAT	2318
131		131
2319	AGACTAATATAGAGTGATCAAAGAACTGAGGTTCTGAAAGAGGCGTGAAGTCGCGTTGT	2378
131		131
2379	GACTCCCTTTGCCATGTTGGGAAGTGTGGCTCAACATTGTGTTTCAGGTTTGCTCAGGGTG	2438
131		131
2439	ATNTCGAACTGACGTNTTGATGAGGGTTATTGC.....	2471
131		131

FIG.1D

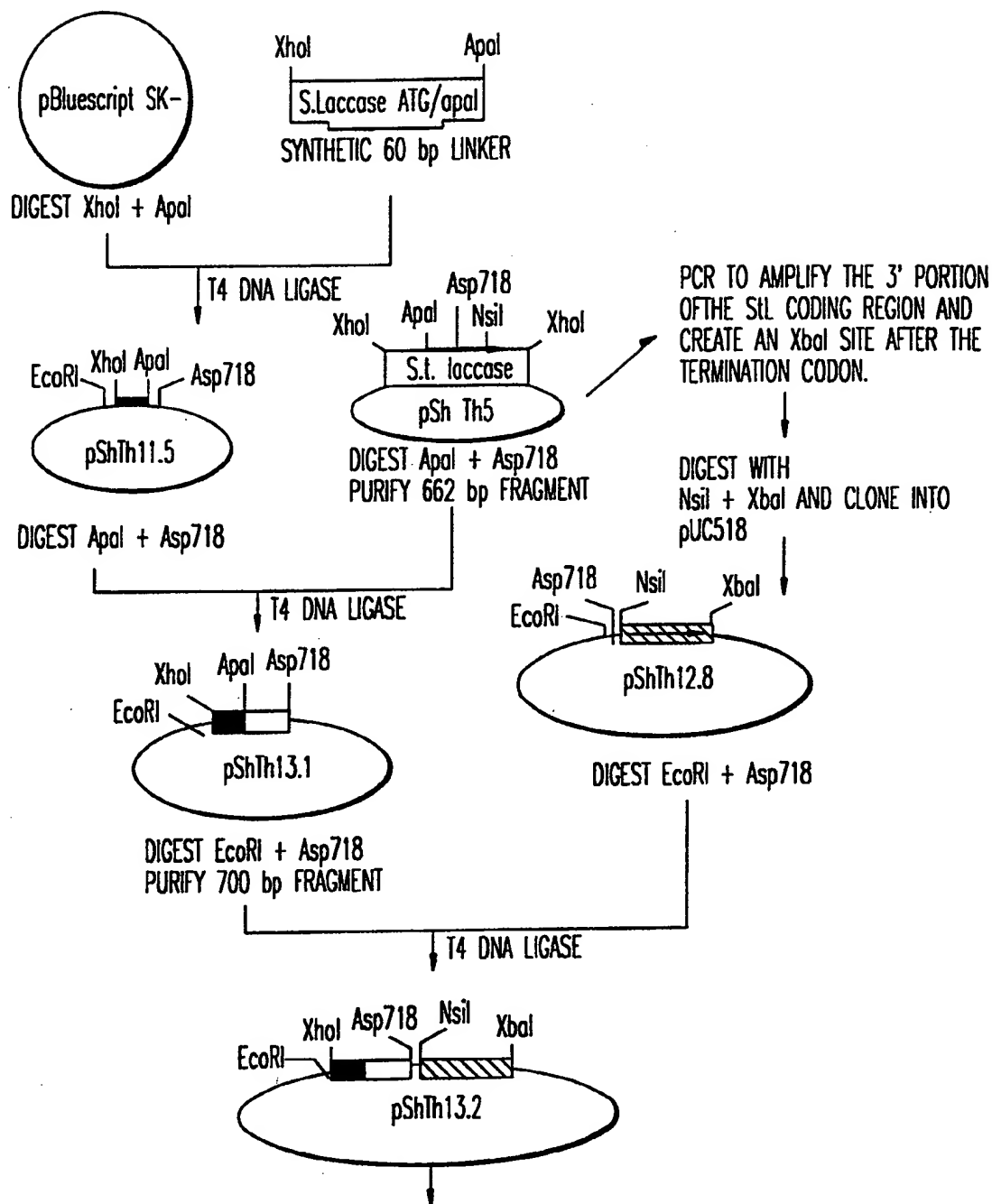


FIG.2A

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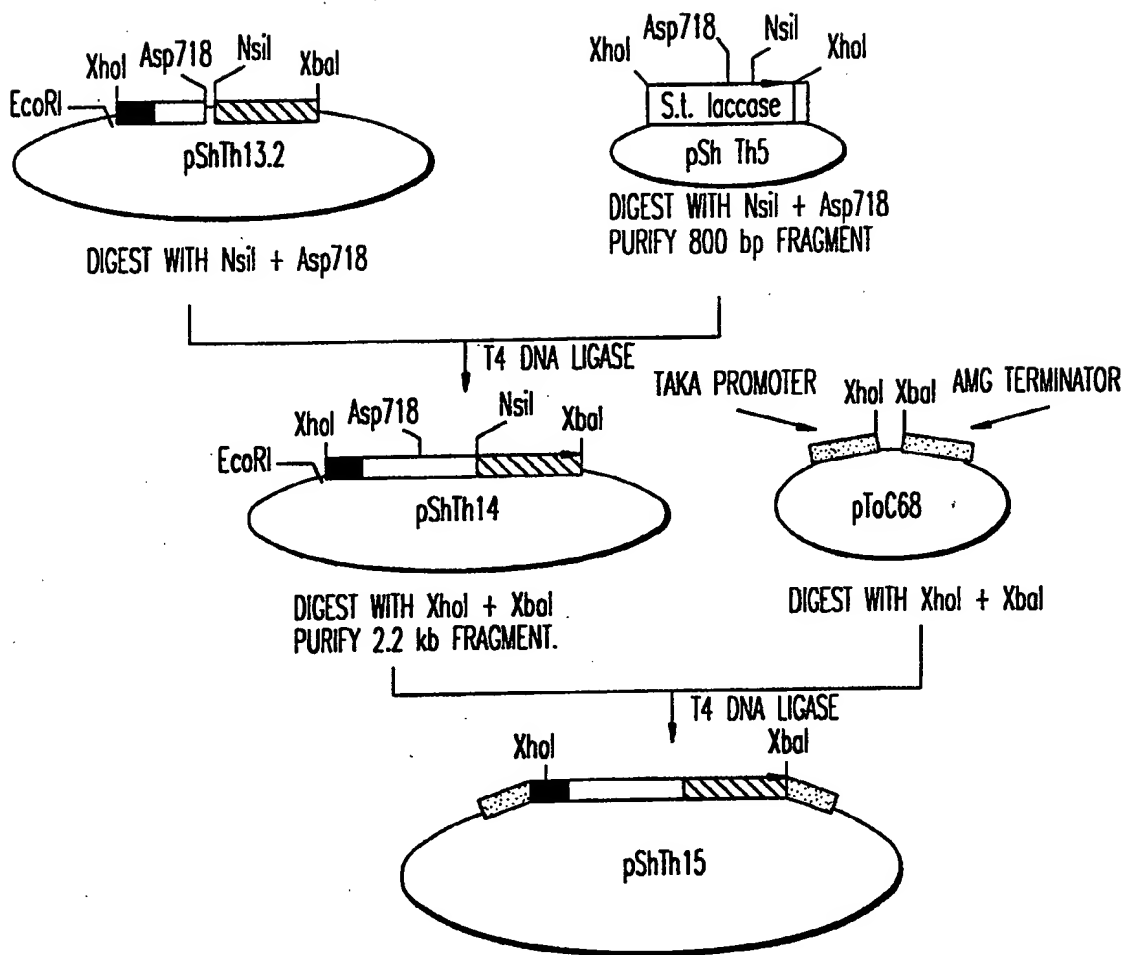


FIG.2B

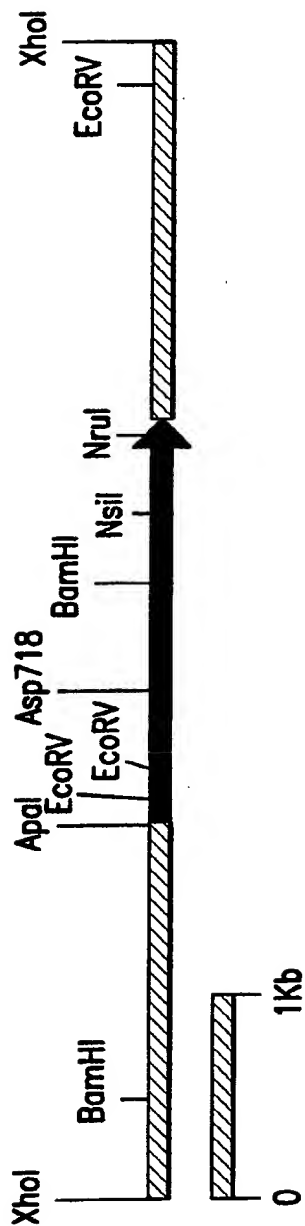


FIG.3

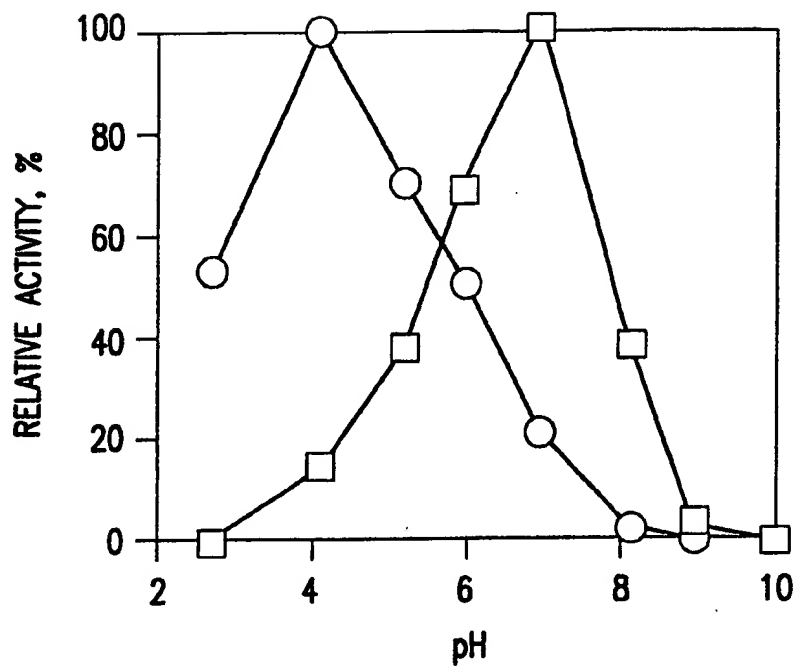


FIG.4

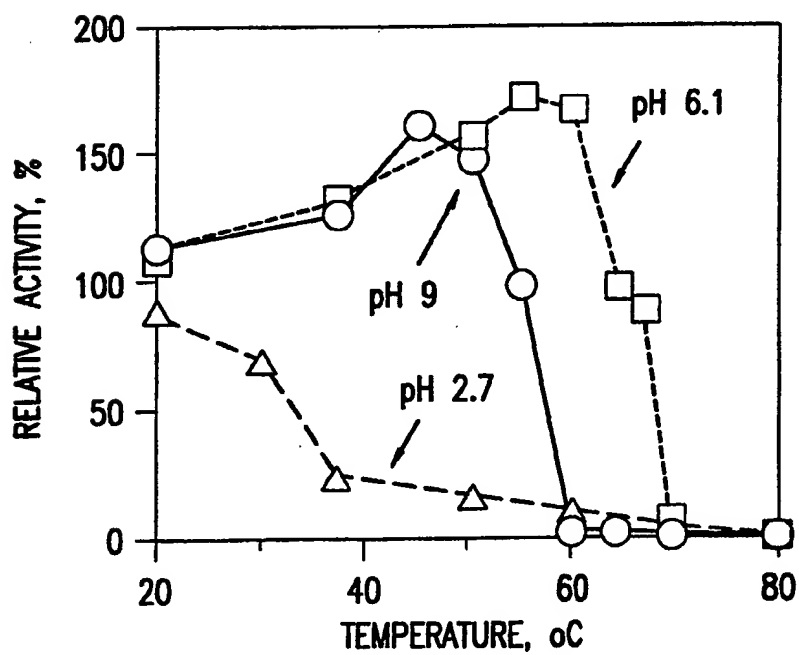


FIG.5

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 95/06816

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N15/53 C12N9/02 C12N1/15 C12N1/38 A61K7/13
 A61K7/06 D21C5/00 //(C12N1/15,C12R1:66)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C12N A61K D21C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, O, X	ABSTRACTS OF PAPERS, vol.209, no.1-2, April 1995 BERKA R. ET AL. 'Cloning of laccases from the thermophilic fungi Myceliophthora thermophila and Scytalidium thermophilum and their heterologous expression in Aspergillus oryzae' see BIOT 196	1,2,6,7, 9,10, 18-20, 24,25
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol.263, no.2, 1988, BALTIMORE, MD US pages 885 - 896 GERMANN U. ET AL. 'Characterization of two allelic forms of Neurospora crassa laccase' see the whole document	

☐ Further documents are listed in the continuation of box C.☐ Patent family members are listed in annex.

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 "&" document member of the same patent family

Date of the actual completion of the international search

29 August 1995

Date of mailing of the international search report

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